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Degradation of MDM2

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13. ABSTRACT (Maximum 200 Words) The interaction between mdm2 and p53 is a viable therapeutic target, as overexpression of mdm2 can lead to excessive p53 degradation, suppressing a cell's ability to cope with cellular insult. The goal of this research is to use recent advances in nanotechnology to develop a specific nanoparticle antagonist to disrupt the mdm2:p53 interaction. Inhibiting the interaction between p53 and mdm2 allows wild-type p53 concentrations to rise to functional levels, effectively killing proliferating tumor cells. By incorporating traditional peptide inhibitors of mdm2 with mixed monolayer protected gold cluster nanoparticles, we hope to effect mdm2 denaturation on the nanoparticle surface, increase peptide stability, and facilitate intracellular peptide delivery. Nanoparticle characteristics, such as size, surface chemistry and biocompatibility, may be controlled and modified for these specific applications. In the first year of the research grant, nanoparticles featuring mdm2-specific peptides have been synthesized and characterized by transmission electron microscopy, gel electrophoresis, UV-Vis absorbance, and fluorescence spectrometry. The nanoparticles are water soluble, stable for months at room temperature, and successfully inhibit the binding of mdm2 to p53. Current work is focused on optimizing peptide loading on the nanoparticle, determining changes in mdm2 conformation upon nanoparticle binding, and preparing for experiments on cultured cells.				
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Introduction

The interaction between mdm2 and p53 is a viable therapeutic target. Overexpression of mdm2 can lead to excessive degradation of p53, suppressing a cell's ability to cope with cellular insult.¹ Mdm2-conferred tumorigenicity has been implicated in a number of human tumors, including breast cancer.²⁻⁵ Disruption of the mdm2:p53 interaction enables functional levels of p53 to accumulate, allowing cells to either halt cell division or initiate apoptosis.⁶ Numerous studies have been successful in disrupting the mdm2:p53 interaction using inhibitory peptides,^{7,8} peptide analogs,⁹⁻¹¹ or polycyclic compounds.¹²⁻¹⁶ These inhibitors target the deep hydrophobic cleft of mdm2 that interacts with the N-terminal α -helix of p53. Although promising, many of these approaches have inherent challenges ranging from stability in cellular environments to cellular delivery. Based on these limitations, an approach integrating specificity, stability, internalization and biocompatibility is needed. The advent of nanoparticle technology may present an ideal way to address these issues.

Body

Our group has demonstrated the versatility of nanoparticles in biological settings, ranging from plasmid transfection of mammalian cells to tunable binding of protein surfaces.¹⁷⁻²⁰ These studies validate the biological application of nanoparticles and suggest that they can be used to inhibit mdm2. The disruption of the p53:mdm2 interaction can rescue cells that are characterized by wild type p53 and overexpressed mdm2. Nanoparticles functionalized with a previously studied p53 peptide⁷ will be used to specifically bind mdm2. Upon binding, mdm2 may denature on the surface of the nanoparticle, removing the potential for release and further action on p53. Given the relative size and surface functionality, each nanoparticle will be able to bind multiple copies of mdm2. This provides an efficient means of effectively decreasing intracellular mdm2 concentrations, allowing p53 to reach wild type levels, thus enabling a cellular approach to tumor elimination.

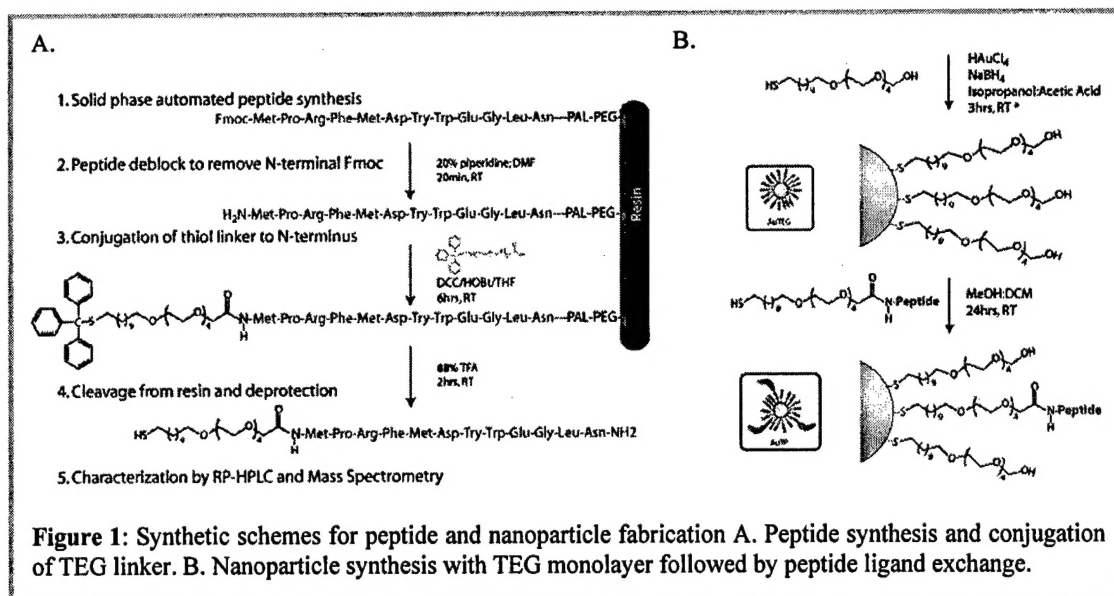
Initial efforts focused on 1) expression and purification of recombinant mdm2 and p53, 2) synthesis of peptide-tagged nanoparticle, and 3) validation of the proposed hypothesis in an *in vitro* setting. Optimization of the peptide and nanoparticle composition could then be conducted.

1) Expression and purification of recombinant mdm2 and p53:

My early attempts to express mdm2 and p53 relied on generating recombinant proteins incorporating a poly-His tag to facilitate purification and immobilization. Unfortunately, both yield and purity of these recombinant proteins were poor. Based on the cited success of GST-tagged mdm2 and p53,⁸ these clones were either obtained or synthesized. Both GST constructs feature thrombin-cleavable linkers, allowing for selective removal of the GST tag, an important requirement for subsequent binding assays. Purification of both constructs involves an initial glutathione-resin based column, followed by anionic exchange FPLC. Initial binding studies were successful, indicating that the proteins are in native, active conformations

2) Synthesis of peptide-tagged nanoparticles:

For these studies, the goal was to generate nanoparticles that were decorated with poly(ethylene glycol) to protect the nanoparticle from nonspecific biomolecule binding and to force the peptide to extend into solution (as opposed to binding to the particle surface).



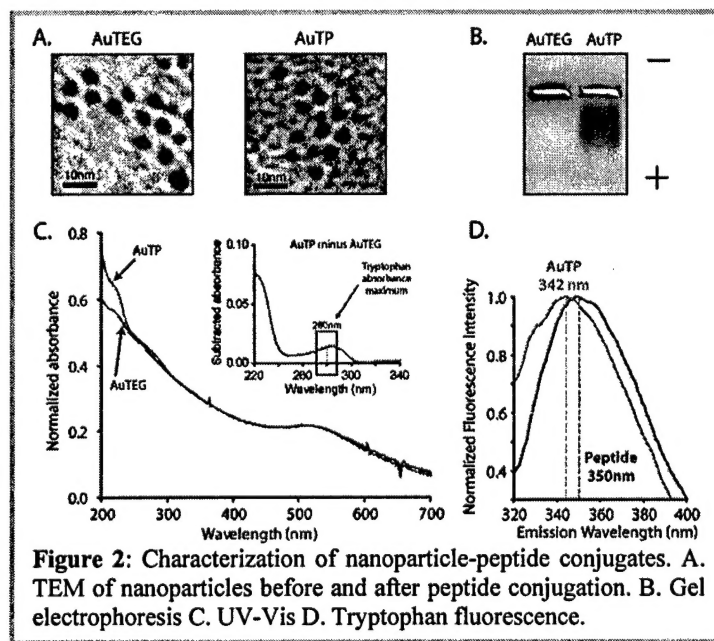
The peptide, conjugated to a thiol-tetra(ethylene glycol) (TEG) linker, would then be displaced onto the nanoparticle surface, relying on the method developed by Hostetler *et al.*²¹ Requirements for this approach are 1) a peptide functionalized with a thiol-TEG linker and 2) TEG-capped monolayer protected gold clusters.

Peptide synthesis: Peptide synthesis was conducted using solid-state Fmoc chemistry on an automated synthesizer. For peptide attachment to the nanoparticle, it was necessary to conjugate a thiol-terminated alkane-PEG linker to the N-terminus of the peptide (Figure 1A). The N-terminal acetylated peptide was also prepared as a control. Peptide identity was characterized by MALDI-TOF mass spectrometry.

Nanoparticle synthesis:

Tetra(ethylene glycol)-capped nanoparticles were prepared as described,²² resulting in water soluble gold-core nanoparticles (AuTEG) with core diameters of 3 nm (± 1 nm). Purified nanoparticles were incubated with excess linker-functionalized peptide to effect peptide incorporation onto the nanoparticle (Figure 1B). This synthesis successfully resulted in peptide-tagged nanoparticles (AuTP) which remained water soluble and stable for months at room temperature.

AuTP nanoparticles were purified from free peptide and



ligand by size exclusion chromatography followed by extensive dialysis against distilled, deionized water. The peptide incorporation onto the nanoparticle surface was determined by NMR. By comparing the signal of aromatic protons to those contributed by the alkane and TEG moieties, it was possible to calculate a peptide coverage of 14.7%. Using values calculated by Hostetler *et al.*,²³ we were able to determine a molecular weight for the average nanoparticle (with core sizes of 3nm) to be 309kDa. These calculations are required to compare efficacy of AuTP vs. free peptide in the disruption of the mdm2-p53 interaction. Initially, nanoparticles were characterized by transmission electron microscopy (TEM) to verify that ligand exchange has no effect on core size and shape (Figure 2A). Peptide incorporation was determined by gel mobility assay (Figure 2B). AuTEG showed no mobility since the TEG surface is charge neutral. The AuTP nanoparticles, however, have a slight negative charge, resulting in significant mobility. Furthermore, AuTP nanoparticles displayed characteristic peptide peaks (220 and 280 nm) by UV-Visible absorbance spectroscopy (Figure 2C). In addition, fluorescence spectroscopy was used to determine the presence of the tryptophan residue on the AuTP surface (Figure 2D). The Trp emission maximum around 350 nm indicates that the peptide is in a polar environment, suggesting extension into solution rather than binding to the nanoparticle surface.

3) AuTP-mediated inhibition of mdm2:p53 interaction:

To determine the inhibitory effects of the AuTP nanoparticle on the mdm2-p53 interaction, an ELISA was performed (Figure 3). To ensure that multiple copies of mdm2 are bound by the nanoparticle-bound peptides, p53-GST was immobilized on the glutathione coated 96-well plate. AuTEG particles had no effect on the interaction of mdm2 and p53. As expected, the peptide alone exhibited an IC_{50} of 252 ± 28 nM, concurrent with the cited value of 300 nM.⁷ The AuTP IC_{50} , based on the overall nanoparticle MW, was determined as 53.6 ± 12 nM. Based on the peptide fraction of the nanoparticle, the IC_{50} was 1420 ± 340 nM. These results are very promising, in that they clearly demonstrate that peptides tagged to the nanoparticle surface retain intrinsic activity. Furthermore, the TEG periphery of the monolayer is sufficient in prohibiting interactions with either mdm2 or p53 due to non-specific binding.

The efficacy of the AuTP mediated inhibition can be improved on various fronts.

1. Decrease in peptide coverage on nanoparticle will increase binding efficiency (if determinations are based on protein fraction of molecular weight). By maintaining a low peptide coverage

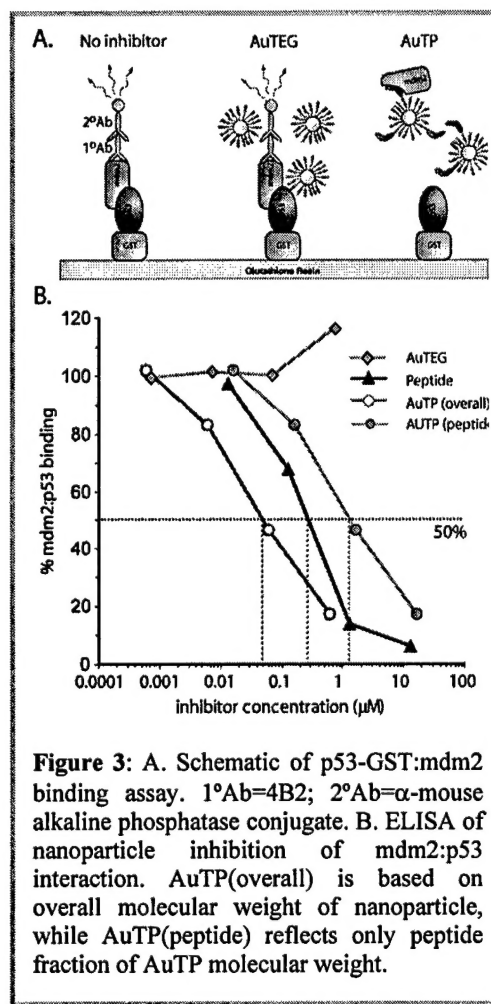


Figure 3: A. Schematic of p53-GST:mdm2 binding assay. 1°Ab=4B2; 2°Ab= α -mouse alkaline phosphatase conjugate. B. ELISA of nanoparticle inhibition of mdm2:p53 interaction. AuTP(overall) is based on overall molecular weight of nanoparticle, while AuTP(peptide) reflects only peptide fraction of AuTP molecular weight.

on each nanoparticle, steric hinderances upon mdm2 binding can be avoided, enabling every peptide to bind the target protein.

2. Experimental determination of peptide loading can be improved. Current use of NMR to determine loading is difficult and imprecise. A combination of TEM, elemental analysis, thermo-gravimetric analysis, and UV-Vis absorbance can be used to obtain more precise loading values.

3. The poor water solubility of the peptide-linker conjugate makes characterization and purification more difficult. Zhang *et al* have recently characterized a modified mdm2-binding peptide (F-K-K-Ac₆c-W-E-E-L).²⁴ This peptide is highly water soluble and can more easily form the α -helix required for binding. Preliminary tests with this peptide are presently underway.

Upon addressing the nanoparticle improvements outlined above, characterization of the AuTP:mdm2 interaction will continue. The time-dependence of AuTP-mediated disruption of the mdm2-p53 interaction will be studied, as well as the effects of AuTP on the conformation of mdm2 (as monitored by circular dichroism). In addition, studies in cultured mammalian cells will begin. To obtain preliminary data on nanoparticle uptake, confocal microscopy was used to determine localization of positively charged nanoparticles labeled with a fluorescence marker (Figure 4). Although this nanoparticle differs from AuTP, the required cell culturing and microscopy skills have been obtained.

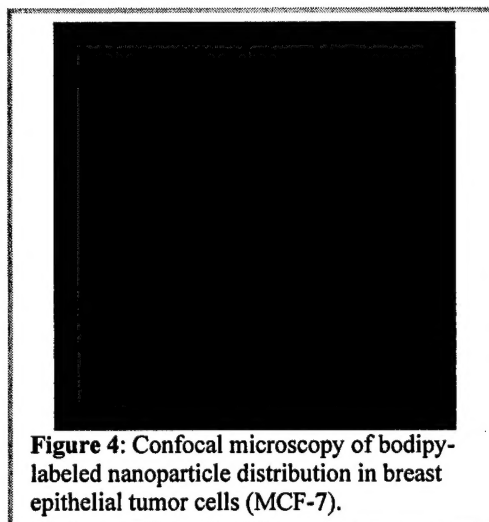


Figure 4: Confocal microscopy of bodipy-labeled nanoparticle distribution in breast epithelial tumor cells (MCF-7).

Key Research Accomplishments

- Expression and purification of mdm2 and p53 with cleavable GST tags
- Synthesis of peptide and peptide-linker conjugate
- Synthesis of stable and water-soluble peptide-functionalized nanoparticles (AuTP)
- Disruption of mdm2:p53 interaction using AuTP
- Preliminary confocal microscopy studies of nanoparticle uptake

Reportable Outcomes

American Chemical Society National Conference (Philadelphia, PA) Aug 2004. *Nanoparticle-mediated rescue of p53 through targeted binding of mdm2*. (Accepted as poster presentation in Division of Organic Chemistry)

Gordon Research Conference: Drug Carriers in Medicine and Biology (Bozeman, MT) Sept 2004. *Nanoparticle-mediated inhibition through protein surface binding*. (Accepted as poster presentation. Could not attend due to medical emergency)

Conclusions

Initial results have demonstrated that peptide-tagged nanoparticles are stable, water soluble, and capable of disrupting the mdm2:p53 interaction. First-year objectives have been addressed, and optimization of both nanoparticle and peptide characteristics will allow a timely progression to the second-year goals. Changes in the peptide sequence, as outlined in the report body, may help address the difficulties in nanoparticle-peptide characterization. The ability to successfully conjugate a functional peptide to the nanoparticle illustrates the potential use of peptide-tagged nanoparticles as vectors for targeted therapy.

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